Cytoplasmic transfer in oocytes: biochemical aspects

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Cytoplasmic control of preimplantation development is not a ‘new’ concept; the first cytoplasmic transfer experiment was performed in the mouse during the early 1980s, as a means of overcoming cleavage arrest at the 2-cell stage, the ‘2-cell block’. Since the first human pregnancy following the transfer of cytoplasm from donor oocytes into the oocytes of a patient with a history of poor embryo development and recurrent implantation failure in 1997, >30 children have been born after direct injection of ooplasm from fresh, mature or immature, or cryopreserved–thawed donor oocytes into recipient oocytes via a modified ICSI technique. Transfer of ooplasm was thus applied with astonishing speed in humans, in the absence of extensive research to evaluate the efficacy and the possible risks of the method. This review focuses on biochemical mechanisms by which transfer of ooplasm might confer a benefit: by correcting a putative imbalance between anti- and pro-apoptotic factors and/or correction of defective mitochondrial membrane potential. We also emphasize the ‘empirical’ state of this technique, and the related risks.

Key words: apoptosis/epigenetic risk/ooaplasm transfer

Introduction

Cytoplasmic control of preimplantation development is not a ‘new’ concept; the first cytoplasmic transfer experiment was performed in mouse during the early 1980s, as a means of overcoming cleavage arrest at the 2-cell stage, the ‘2-cell block’. Transfer of cytoplasm from embryos without cleavage arrest to those from strains that did arrest alleviates this 2-cell block, allowing the embryos to continue development in vitro (Muggleton-Harris and Whittingham 1982). In 1997, Cohen et al. announced the first human pregnancy following the transfer of cytoplasm from donor oocytes into the oocytes of a patient with a history of poor embryo development and recurrent implantation failure. The live birth of five healthy children was reported by the end of 1998 (Cohen et al., 1998), and this method was then proposed as a means of restoring some unknown defects in oocytes from patients with repeated implantation failure, using a bolus of donor cytoplasm via the transfusion of unidentified factors (mRNA or proteins) into the recipient defective oocyte. Since then, >30 children have been born after direct injection of ooplasm from fresh, mature or immature, or cryopreserved–thawed donor oocytes into recipient oocytes via a modified ICSI technique (Cohen et al., 1997, 1998; Lanzendorf et al., 1999; Huang et al., 1999; Dale et al., 2001; Hwang et al., 2002). Transfer of ooplasm was thus applied with astonishing speed in humans, in the absence of extensive research to evaluate the efficacy and the possible risks of the method. As a result, the potential dangers (De Rycke et al., 2002; Templeton, 2002; Winston and Hardy, 2002) and unpredictable outcomes (Cummins 2001, 2002) of this technique have been highlighted in recent publications.

Following ovulation, zygote survival depends almost exclusively on maternal mRNA and proteins that accumulate during oocyte growth and maturation, until the stage where the new zygote genome is activated, the maternal-to-zygotic transition (MZT), which happens during the 4–8-cell stage in humans (Braude et al., 1988). Maternal transcripts are thus responsible for the first few cleavage divisions and for transition of the maternally controlled zygote into an activated embryonic genome (Latham, 1999). Therefore, the quality of the oocyte cytoplasm, whether or not manipulated, will establish the future of the embryo. This review focuses on biochemical mechanisms by which transfer of ooplasm might confer a benefit: by correcting a putative imbalance between anti- and pro-apoptotic factors and/or correction of defective mitochondrial membrane potential. We also emphasize the ‘empirical’ state of this technique, and the related risks, such as mitochondrial heteroplasmy, mitochondrial disease and nuclear–mitochondrial interaction, epigenetic aspects, and potential influence on regulatory protein polarization and on aspects of mRNA polyadenylation.

Apoptosis

During the human life span, >99.9% of our cells undergo a physiological suicide process known as apoptosis (Vaux et al.,
1999). This cell death is defined as a process that follows an intrinsic genetic program, activated at a controlled time. Apoptosis is commonly used to control cell number and maintain homeostasis, acting as a defensive strategy by removing mutated, damaged, superfluous, unwanted or useless cells. Recently, its role in gamete maturation and early embryogenesis has been highlighted (Tilly, 2001, 2003; Levy, 2001; Russell et al., 2002), and morphological and biochemical markers of apoptosis have been described in the mammalian pre-embryo (Jurisicova et al., 1995, 1996; Levy et al., 1998; Brison, 2000; Hardy et al., 2001). Unlike cell death due to necrosis, apoptosis strictly affects individual cells, with morphological features that include condensation of chromatin at the nuclear membrane, membrane blebbing (without loss of integrity), shrinking of cytoplasm leading to the loss of intercellular connections, and finally the formation of membrane-bound vesicles (apoptotic bodies) (Wyllie et al., 1980; Sanders and Wride, 1995). Caspase substrates can regulate the key morphological changes in apoptosis. Several caspase substrates also act as transducers and amplifiers that determine the apoptotic threshold and cell fate (Fischer et al., 2003). Mitochondria exert a decisive role in cell death control through the release of pro-apoptotic factors in the cytoplasm, cytochrome C and apoptosis-inducing factor (AIF), leading to the activation of a caspase cascade together with changes in membrane asymmetry and exposure of phosphatidylserine (PS) on the membrane. In considering a potential benefit of cytoplasmic transfer, the impact on the recipient oocyte should be considered in terms of balance between pro- and anti-apoptotic factors (for review, see Levy, 2001). This balance is regulated by both intrinsic and extrinsic factors.

**Intrinsic factors**

DNA fragmentation has been reported in mouse gonadotrophin-stimulated atretic follicles and oocytes (Perez et al., 1999). Similar results were observed in human ovarian follicles obtained after oocyte retrieval from patients undergoing IVF. Controlled ovarian stimulation is routinely used in clinical practice in order to obtain a large number of oocytes, and this can sometimes lead to an excessive follicular response to administered gonadotrophins. This can potentially result in inadequate biochemical maturation of oocyte cytoplasm. Oocytes with such a deficiency could have an unbalanced apoptotic system that might jeopardize the processes of fertilization and subsequent development. In support of this hypothesis, an increasing number of highly variable apoptosis-related transcripts has been detected during different stages of murine and human oocyte development (Jurisicova et al., 1998, 2003; Exley et al., 1999; Kawamura et al., 2003). The quantity, and in particular the quality, of these transcripts is clearly a crucial element for successful fertilization and embryo development. Although it is not routinely possible to test each oocyte for apoptotic transcripts before IVF, the different pathways that regulate control of apoptosis in the oocyte have been extensively studied.

**Oral antioxidants**

A recent study analysed the effect of oral antioxidants on both the number and the quality of oocytes retrieved from aged mice after exogenous ovarian stimulation (Tarin et al., 2002). Interestingly, the negative effects of female aging in oocytes retrieved from oviducts and ovaries were counteracted by both early and late administration of oral antioxidants, as assessed by number of oocytes and percentage with normal metaphase II chromosome distribution and/or morphological evidence of apoptosis. Despite this apparently beneficial effect, oral antioxidant administration is a non-specific approach to regulation of apoptosis in oocytes. Application of these data in clinical IVF should be made with caution, but it might be worth of consideration prior to the more aggressive approach of cytoplasmic transfer. A more physiological approach might be via manipulation of culture systems for *in vitro* oocyte maturation (IVM) but so far this technique has had a relatively low success rate compared with conventional IVF/ICSI.

**Growth hormone**

Recent evidence suggests that growth hormone (GH) is involved in ovarian regulation and may act as a survival factor during *in vitro* culture, reducing apoptosis by altering the bax:bcl-2 ratio during early embryogenesis (Kolle et al., 2002). These authors analysed the mechanisms of GH action during IVM of bovine cumulus–oocyte complexes (COC) (Kolle et al., 2003): COC matured *in vitro* in the presence of GH revealed a significantly (*P < 0.05*) higher proportion of proliferating cumulus cells, and GH treatment significantly reduced (*P < 0.05*) apoptosis in the cumulus cells as determined by terminal deoxynucleotidyl transferase-mediated dUDP nick-end labelling (TUNEL) assay. These data suggest that GH increases cumulus expansion by promoting cell proliferation and inhibiting apoptosis. We have recently detected the GH receptor in naked oocytes as well as in COC (Ménézo et al., 2003). Thompson et al. (2000) demonstrated that GH can increase the capacity of liver cells to repair DNA, and by analogy we hypothesize that a similar mechanism might occur in the female gamete: GH could upgrade oocyte quality by increasing its DNA repair capacity. This is clearly an important process at the time of fertilization and during early embryogenesis (especially in ICSI). In parallel, new insights have emerged over the past few years about the role of ceramide and sphingosine-1-phosphate (S1P) as mediators and suppressors, respectively, of cancer therapy-induced oocyte apoptosis. These studies have allowed the development of novel lipid-based strategies to combat apoptosis-related infertility and premature menopause in female cancer patients (Casper and Jurisicova, 2000; Morita et al., 2000; Spiegel and Kolesnik, 2002; Tilly and Kolesnik, 2002). Such aetiological strategies for restoring apoptotic balance might provide a more elegant approach to improving oocyte cytoplasm quality than oocyte microsurgery.

**Apoptotic gene expression**

An understanding of the genes responsible for regulating apoptosis is crucial in order to elucidate the mechanisms involved and the reasons why it occurs. Recent data report that mammalian oocytes and preimplantation embryos possess abundant levels of transcripts encoding cell death suppressor and inducer genes, and caspase genes were also detected during all developmental stages. Significant differences in apoptosis-related mRNA between non-frAGMENTED and fragmented mouse and human embryos were also demonstrated, suggesting that cellular fragmentation in a subset of human preimplantation embryos could be regulated by certain components of a genetically programmed cell death (Van Blerkom et al., 2001; Jurisicova et al., 2003). However, Martinez et al. (2002) also suggest that caspases in preimplantation human...
embryos are involved in developmental processes unrelated to cell death, and therefore data regarding specific gene expression should be interpreted/extrapolated with caution.

Apoptosis plays an active and crucial role in the developing embryo through the removal of deficient cells (Hardy, 1999), and must be considered as a normal feature in both in vivo and in vitro preimplantation development. However, apoptosis may also have detrimental effects if either the number of apoptotic cells or ratio of these cells to the normal cells exceeds a critical threshold. This could occur both in suboptimal culture conditions in vitro and during in vivo compromised development. Jurisicova et al. (1998) suggest a model in which embryo development is regulated by a balance of pro- (i.e. Bax) and anti- (i.e. Bcl-2) apoptotic gene expression with successive checkpoints, from the time of oogenesis throughout the preimplantation period. Mature oocytes with appropriate anti-apoptotic mRNA will succeed through maturation and fertilization, whereas poor quality oocytes with high levels of cell death inducers or low levels of cell death antagonists will fail to fertilize, or fragment and/or arrest.

An elegant mathematical model demonstrates that this apoptotic ratio seems to be programmed at an early stage in the zygote, and suggests that once apoptosis has been triggered, the process cannot be disrupted or reversed. This underlines the importance of healthy gametes as the basis for fertilization and successful healthy embryo development (Hardy et al., 2001), and also implies that addition of a 10–15% transfer of ooplasm is unlikely to adequately correct the balance between death promoters and death inhibitors.

**Extrinsic factors**

**Culture conditions**

Levels of cell death in several species in vitro were found to be significantly higher than in vivo (Papaioannou and Ebert, 1986; Brison and Schultz, 1997; Jurisicova et al., 1998). In vitro production also disturbs the chronology of apoptosis in bovine embryos (Gjorret et al., 2003); this is in full agreement with observations that gene activation can be influenced by culture conditions (Niemann and Wrenzycki, 2000). There is controversy surrounding the effects of fetal calf serum supplementation on the development of bovine embryos (Byrne et al., 1999); supplementation of culture media with amino acids has been shown to benefit preimplantation embryo development in several species (Devreker et al., 2001), and restriction of sulphur-free amino acids induces apoptosis (Kang et al., 2002; Lu et al., 2003). Glucose and insulin concentrations adversely affect rat blastocyst development both in vivo and in vitro (Pampfer et al., 1997; Pampfer, 2000; Jimenez, 2003), particularly at the blastocyst stage, where there was an association with a higher degree of apoptosis in the inner cell mass in vitro.

**Growth factors**

Growth factors are known to regulate cell proliferation and differentiation during mammalian preimplantation development, some also acting as survival factors (Hardy and Spanos, 2002). Supplementation of culture medium with exogenous growth factors such as transforming growth factor-β (TGF-β) partially reduced excessive in vitro apoptotic cell death without accelerating development or increasing final cell number (Brison and Schultz, 1997, 1998). Insulin-like growth factor I (IGF-I) was recently confirmed to have a clear anti-apoptotic action during blastocyst development (Herrler et al., 1998; Spanos et al., 2000). Platelet-activating factor (PAF; 1-o-alkyl-2-acetyl-sn-glycero-3-phosphocholine), a potent ether phospholipid, also acts as an autocrine growth/survival factor for the mammalian preimplantation embryo (O’Neill, 1997; Stojanov and O’Neill, 1999).

**Air pollution and smoking**

Germline cells are vulnerable to genetic damage from smoking, apparently by altering the meiotic spindle of oocytes and sperm, leading to chromosome errors that affect reproductive outcomes (Zenzes, 2000). An understanding of the mechanisms involved could help in the development of potential corrective intervention. Polycyclic aromatic hydrocarbons (PAH), found not only in cigarette smoke but also in polluted air, interact with the aryl hydrocarbon receptor (Ahr) and induce toxicity mediated by a Bax pathway (Zenzes, 2000; Matzuk, 2001). Cadmium nicotine and its metabolite, cotinine, are detectable in gonadal tissues and fluids in association with smoking; cotinine is incorporated into ovarian granulosa-lutein cells, with the potential to compromise the follicle’s developmental potential. Smoking-related effects are detectable in ovarian granulosa-lutein cells, oocytes, sperm and preimplantation embryos. The damage caused by these factors might be repaired during meiosis in the germinal cells, but ejaculated sperm have a very limited capacity for repairing damage: smoking is associated with low sperm quality. Transmission of altered DNA by sperm (but not yet by oocytes) was demonstrated in preimplantation embryos, and paternal preconception smoking has been linked to a significantly elevated risk of childhood cancers, particularly acute leukaemia and lymphoma (Ji et al., 1997). If damage has already occurred in the ejaculated sperm, addition of external cytoplasm to an oocyte is unlikely to change the poor prognosis for embryo development caused by the DNA-damaged sperm. Finally, several studies suggest that cotinine can inhibit fragmentation (and apoptosis) in developing embryos of smokers, and this acquisition of resistance to fragmentation via smoking may confer a dangerous survival advantage to genetically altered embryos (Zenzes, 1999, 2000). In this case, external addition of healthy cytoplasm might confer an increased capacity for DNA repair, if the transplanted bolus contains the necessary repair enzymes, such as polymerase beta or the multifunctional DNA repair enzyme APEX. Murdoch and Van Kirk (2001) have demonstrated that polymerase beta is upgraded by estrogens, and we were also able to demonstrate the presence of APEX mRNA in human oocytes. Since a high capacity for DNA repair is clearly necessary during the first cycle of DNA replication, when pronuclei are formed, the presence of mRNA coding for DNA repair enzymes could be considered as a marker of oocyte quality. In borderline ‘aged’ oocytes, the addition of extra ‘good’ cytoplasm could thus enhance the prognosis for obtaining an ongoing pregnancy.

**Oxidative stress**

The generation of reactive oxygen species (ROS) is a factor of major importance: ROS are generated by several different metabolic pathways, including oxidative phosphorylation, NADPH oxidase and xanthine oxidase systems. ROS generation is enhanced during in vitro culture, especially at the time of genomic activation (Nasr-Esfahani et al., 1990; Johnson and
Nasr-Esfahani MH, 1994). There are at least two key substrates that exert a major influence on metabolism in in vitro culture conditions, glucose and sulphur amino acids, including methionine. Excessive glucose levels induce apoptosis, by increasing the level of Bax, a pro-apoptotic factor, and by influencing the expression of caspase 3 and caspase-activated deoxyribonuclease (CAD) (Pampfer, 2000; Pampfer et al., 2001; Leunda-Casi et al., 2002). In murine and bovine systems, hyperglycaemia affects sex ratio through apoptosis, related to the production of X-linked inhibitor of apoptosis protein (XIAP) (Jimenez et al., 2003).

An imbalance in oxidative stress can also induce apoptosis (Korsmeyer et al., 1993, 1995). In preimplantation human embryos, both H2O2 concentration and DNA fragmentation levels are higher in fragmented than in non-fragmented embryos (Yang et al., 1998). We recently demonstrated (Oger et al 2003) that sperm DNA fragmentation is also linked to ROS. Over a certain limit of fragmentation, DNA cannot be repaired or will be repaired with a high level of aberrations, thus leading to possible mutations. In the oocyte, the oxidation product of sperm DNA, 8-oxo-guanine, may be removed from oxidatively damaged DNA by classical base excision repair (Bessho et al., 1993). When base excision repair is absent or drops below a critical threshold, mistakes are introduced, with G:C replaced by T:A. This mutation may lead to carcinogenesis (Boiteux and Radicella 1999, 2000). On this basis, addition of exogenous high quality cytoplasm that boosts DNA repair systems could provide an advantage for further embryonic development.

Can apoptosis in the preimplantation embryo be prevented? There is increasing evidence that human development is regulated before implantation by factors derived from both the oocyte and the embryo, including growth factors and apoptosis-related genes. Ooplasm donated from a healthy oocyte is presumed to provide specific endogenous survival growth factors, mRNA and/or appropriate anti-apoptotic mRNA to restore the balance of apoptosis-related mRNA. Recurrent high levels of fragmentation and poor embryo quality and development have been an indication for cytoplasm donation, and it is of interest to note that the pregnancies reported after cytoplasmic transfer (CT) were associated with a significant reduction in embryo fragmentation (Lanzendorf et al., 1999; Dale et al., 2001). However, CT did not enhance success in women with advanced reproductive age or poor ovarian reserve; in these cases there was no improvement in embryo quality, and pregnancies resulted in biochemical losses and aneuploid clinical loss (Opsahl et al., 2002). Transfer of ooplasm can also introduce hazardous elements, by increasing the level of apoptosis in the embryo. Since the process of apoptosis in the preimplantation embryo is not fully elucidated and the anti- or pro-apoptotic factors have not yet been fully identified, CT is applied on a solely empirical basis. Progress in molecular technology has greatly increased our knowledge about the number of known related apoptosis genes expressed in the oocyte and during embryonic development. Quantitative and real-time RT–PCR or a microarray methodology applied mRNA phenotyping could provide helpful tools to investigate the nature of the transferred cytoplasmic elements. Survivin is a member of the IAP (inhibitors of apoptosis) family that can bind to caspases and modulate their function. This factor is present at the mRNA and protein level in the preimplantation embryo, and is a potential candidate as one of the anti-apoptotic factors (Kawamura et al., 2003).

Although preventing apoptosis might appear to be beneficial, the potential effect of its prevention on further embryo development must also be considered. For example, apoptosis is the developmentally acquired phenomenon that occurs in embryos exposed to elevated temperature. Experimental inhibition of group II caspases mediating heat-induced apoptosis in bovine embryos had a clear detrimental effect on embryonic resistance to heat shock. Apoptosis after heat shock can be viewed as an adaptative mechanism, beneficial in allowing embryonic survival and development following stress (Paula-Lopes and Hansen, 2002a,b; Paula-Lopes et al., 2003).

Mitochondria

Correction of defective mitochondrial membrane potential

Healthy mitochondria are essential for accurate chromatid segregation at the time of fertilization and during subsequent mitotic division. Mitochondria in the cytoplasm are responsible for respiratory processes and ATP production (OXPHOS). They also generate ROS, which are deleterious for biological material, including DNA. It has been proposed that women with a history of IVF failure produce poor quality oocytes that may contain ageing mitochondria, resulting in decreased energy production. Chaotic mosaicism has also been related to low mitochondrial potential (Wilding et al., 2003). Since aged or defective mitochondria might result in aberrant chromosomal segregation or developmental arrest, injection of a bolus of cytoplasm from healthy donor oocytes could potentially restore global mitochondrial activity and rejuvenate recipient oocytes from women with recurrent IVF failure (Wilding et al., 2001).

In addition to their function in cellular respiration, mitochondria also have a central role in the apoptotic-signalling pathway. Cellular anomalies in function at any level are eventually translated into the release of apoptogenic factors from the mitochondrial intermembrane space, resulting in the demise of the cell. These pro-apoptotic mitochondrial factors may contribute to both caspase-dependent and caspase-independent processes in apoptotic cell death (Ravagnan et al., 2002; van Gurp et al., 2003). Mild oxidative treatment has been shown to induce a decline in mitochondrial membrane potential in mouse zygotes, suggesting that oxidative stress induces programmed cell death (PCD), and that mitochondria are involved in the early phase of oxidative stress-induced PCD. Mitochondrial malfunction may thus contribute to cell cycle arrest, triggering mild oxidative stress that is followed by cell death (Liu and Keefe, 2000; Liu et al., 2000). Liu et al. tested this hypothesis by using reconstructed zygotes with nuclei and cytoplasm from H2O2-treated or untreated zygotes, and transfusing healthy mitochondria in order to correct deficient organelles and suppress PCD. Arrested, reconstituted zygotes displayed TUNEL staining indicative of apoptosis at a rate similar to that of H2O2-treated controls, suggesting that apoptotic potential could be transferred cytoplasmically. Reconstituted zygotes derived from H2O2-treated pronuclei mixed with untreated cytoplasm showed significantly increased rates of cleavage and development to morula and blastocyst, indicating that healthy cytoplasm could partly rescue pronuclei from oxidative stress.
Although oxidation had an effect on both nuclei and cytoplasm, cytoplasm was more sensitive to the oxidative stress. This evidence suggests that cytoplasm, and most probably its mitochondrial component, plays a central role in mediating development as well as apoptotic cell death induced by oxidative stress in mouse zygotes.

**Mitochondrial heteroplasmy, mitochondrial disease and nuclear-mitochondrial interaction**

The presence of donor mitochondria was detected in the cells of two out of 15 children at 1 year of age (Barratt et al., 2000, 2001a; Brenner et al., 2000) following the clinical use of cytoplasmic transfer from donor oocytes during IVF treatment. This heteroplasmy has been described as a possible therapeutic effect (Malter, 2002a,b). It has also raised significant concerns regarding the safety of the technique (Brenner et al., 2000). Symptomatic mitochondrial disease due to mtDNA mutation has a very low population frequency (1:8000), and therefore a theoretical risk of mitochondrial disease transmission associated with ooplasmic transfer seems unlikely. It could even be argued that ooplasmic transplantation represents a methodology that might reduce the random transmission of such mitochondrial diseases compared with standard oocyte donation (Malter, 2002a). There is a high degree of mitochondrial homoplasmy throughout the body, and mitochondrial function is controlled by a complex combination of nuclear and mitochondrial genes. Proteins required for mitochondrial function are synthesized in the cytoplasm and imported into the mitochondrial matrix, and survival of mitochondrial genotypes is critically dependent on the nuclear background. The dual nature of this control means that the introduction of ‘foreign’ mitochondrial DNA via ooplasmic transfer can introduce a conflict between the control mechanisms for nuclear DNA, recipient mtDNA and donor mtDNA, leading to unpredictable outcomes (Cummins, 2001). The potential conflict caused by mitochondrial heteroplasmy after fertilization is thought to be one of the evolutionary reasons behind their uniparental inheritance. The elimination of one set of mitochondrial genes at fertilization avoids the possibility of lethal conflict with the nuclear genes, and thus mitochondria pass through a bottleneck during oogenesis or early embryogenesis, with clonal expansion from one or a few of these organelles resulting in potential restoration of homoplasy.

Mitochondria are maternally inherited, so that if the child is female, it is possible that both donor and recipient mitochondria may be transmitted to future generations.

In conclusion, mitochondria are probably the main organelle transferred during injection of cytoplasm, but the manner in which they positively affect embryo rescue, especially their central role in the apoptotic signalling pathway, needs further investigation. The same conclusion may be applied in considering potentially deleterious effects of mitochondrial inheritance after ooplasm transfer in humans.

**Chromosomal abnormalities following ooplasmic transplantation in humans**

Multinucleation is a frequently observed phenomenon in early human preimplantation embryos cultured in vitro, commonly associated with impaired cleavage, poor embryo quality and increased fragmentation, all of which may compromise their implantation potential (Balakier and Cadesky, 1997; Royen et al., 2003). This abnormality in human embryos has been strongly (but not exclusively) correlated with embryo chromosomal abnormalities (Kligman et al., 1996). Multinucleated embryos have a substantially reduced viability, expressed as early cleavage arrest and the formation of abortive blastocysts during in vitro culture; this indicates that the phenomenon is highly lethal. Transfer of cytoplasm from donor oocytes has been proposed as a strategy to rescue these morphologically abnormal embryos.

Follow-up reports of pregnancies and infants born after ooplasmic transplantation reveal that two out of 17 fetuses had an abnormal 45,XX karyotype (Barritt et al., 2000, 2001b). One singleton pregnancy ended in a spontaneous miscarriage in the first trimester with a fetal karyotype of 45,XX, and ultrasonography at 15 weeks gestation confirmed a twin gestation in the second, with one fetus developing abnormally. Amniocentesis performed on the abnormally developing twin revealed a 45,XX karyotype, and this twin was electively reduced. The authors hypothesize that there may be a link between the chromosomal anomalies and oocyte manipulation.

In addition to the two X0 fetuses, one of the babies born was diagnosed at 18 months with ‘pervasive developmental disorder’, one of a spectrum of sex-linked autism-related diagnoses with a high incidence, 1 in 250 children. Whether or not these sex-linked chromosomal anomalies and the mechanism of appearance are linked specifically to cytoplasmic transfer is a matter of controversy.

**Epigenetic aspects**

The possibility that ‘foreign’ cytoplasm might exert an epigenetic effect upon maternal and paternal genomes must also be considered. Different genotypes of mice influence maternal and paternal genomes differently (Baldacci et al., 1992; Reik et al., 1993; Renard et al., 1994; Roemer et al., 1997; Latham and Sapienza, 1998; Pardo-Manuel de Villena et al., 2000; Pickard et al., 2001). Specific manipulations of early mouse embryos result in altered patterns of gene expression and induce phenotypic alterations at later stages of development. Repression and DNA methylation of genes encoding major urinary proteins (MUP), repression of the gene encoding olfactory marker protein, and, interestingly, reduced body weight, can be experimentally induced by nuclear transplantation. Disturbingly, these acquired phenotypes can be transmitted to most of the offspring of manipulated parent mice (Roemer et al., 1997). Recent studies demonstrated that blastomere fragmentation at the 2-cell stage in mouse embryos is under the control of multiple genetic loci, affected by both parental genotypes, possibly as a result of either genomic imprinting or differences in mitochondrial origin (Hawes et al., 2001, 2002). Transfer of cytoplasm could therefore also have immediate adverse effects on early embryo development through blastomere fragmentation and/or apoptosis.

Renard et al. (1994) reported a maternal effect on blastocyst formation. DDK males bred with DDK females are relatively fertile; DDK females cross-bred with males of other strains exhibit reduced fertility, whereas the reciprocal crosses between non-DDK females and DDK males are fertile. The reduction in fertility in the former case results from an incompatibility between the DDK maternal cytoplasm and the non-DDK paternal genome.
expressed during morula-to-blastocyst transition. The active molecule required is transmitted as mRNA that interacts with the paternal genome. Transfer of ooplasm or oocyte RNA from the inbred mouse DDK strain to non-DDK oocytes converts the oocytes to a DDK phenotype, with subsequent post-zygotic arrest. Although it is difficult to speculate on the possibility of a similar situation in humans, it cannot be completely excluded. In such a case, injection of healthy mRNA could not provide any form of rescue for the embryos.

Finally, Pickard et al. (2001) demonstrated that the genotype of the mother can influence the epigenotype of the offspring by a novel epigenetic marking process at fertilization which targets DNA for later methylation in the fetus.

In vitro culture conditions can also alter the DNA methylation imprinting process, mediated by S-adenosyl methionine (SAM). The embryo has an SAM synthase, and SAM is synthesized in the oocyte and in mouse and human embryos before genomic activation (Ménézo et al., 1989). The level of SAM can be disturbed by perturbation of the endogenous methionine pool (Ménézo et al., 1989). Inhibitors of SAM synthesis, as well as deficiency in the endogenous methionine pool, significantly affect embryo development and may even induce apoptosis through deprivation of sulphur amino acids (Kang et al., 2002; Lu et al., 2003). This induces programmed cell death independently of caspase activation, thus increasing apoptosis in the embryo (Son et al., 2001; Kang et al., 2002). Methionine is incorporated by mouse, bovine and human embryos (Ménézo et al., 1989; Guyader-Joly et al., 1997), and both methionine (Guyader-Joly et al., 1996) and cysteine affect glutathione levels and glutathione-related enzyme activities via trans-sulfuration pathways. It is important to note that glutathione is one of the most important factors in preventing the negative effects of ROS, thus reducing apoptosis. Finally, methionine restriction alone may induce cell death through caspase-dependent and -independent pathways (Lu et al., 2003). All of this evidence illustrates that a misunderstanding of embryo metabolism with respect to a single class of amino acids may have a devastating effect on embryo development, especially when oocytes initially have a borderline endogenous pool. Borderline culture conditions can increase apoptosis in the embryo, and this can be avoided by a careful understanding of the biochemical pathways involved. If addition of ‘high quality cytoplasm’ helps to correct the biochemistry, it may be that simpler, more direct approaches might also be effective.

**Polarization of regulatory proteins**

Developmental significance of regulatory protein polarization in early preimplantation embryos

Recent evidence suggests that the differentiation of individual blastomeres may be initiated during very early steps of embryo development. The regulatory protein leptin and the transcription factor STAT 3 displayed polarized distribution in both mouse and human oocytes. After fertilization, these polarized domains become differentially distributed between blastomeres in the cleavage stage embryo and between the inner cell mass and trophoblast of the blastocyst (Antczak and Van Blerkom, 1997). The pattern of inheritance of the polarized domains in daughter blastomeres appears to be associated with the manner in which successive equatorial or meridional planes of cell division interact with the polarized protein domains (Edwards and Beard, 1997). Similarly, considerable variation in the concentration of β-actin and IL-1 mRNA between individual blastomeres originating from the same 6–8-cell stage embryo were noted (Krüssel et al., 1998). This phenomenon of regulatory protein polarization during early human development also exists for transforming growth factor β2 (TGFβ2) and vascular endothelial growth factor (VEGF), the apoptosis-associated proteins Bcl-x and Bax, and the growth factor receptors c-kit and epidermal growth factor receptor (EGF-R) (Antczak and Van Blerkom, 1999).

It has been postulated that the localization of the polarized protein domains to the plasma membrane and subjacent cytoplasm exposes them to membrane extrusions which may occur during fragmentation or biopsy. Loss of non-polarized proteins, such as E-cadherin, has no developmental consequences, whereas spatially limited blastomere fragmentation may deeply alter embryo development if apoptosis proteins (Bcl-x and Bax) exist in a polarized domain.

These findings might have a clinical significance in cytoplasmic transfer. Is the trans fused cytoplasm representative of the donor oocyte as a whole? Does the injection procedure disturb recipient oocyte polarization, with possible detrimental effects on subsequent development? The injection of foreign ooplasm into a crucial polarized domain in the recipient can be hazardous for subsequent embryo development if it alters regulatory protein polarization in the early preimplantation embryos.

**mRNA polyadenylation**

The oocyte and the early preimplantation embryo inherit a stock of mRNA synthesized during oogenesis and the final stages of oocyte maturation; these maternal mRNA drive the first cleavage divisions of the early embryo. At least two known mechanisms are involved in the recruitment of mRNA for translation, the stability of the message and its translation potential. Oocyte maturation in the mouse is linked to a drop in the global quantity of mRNA transcripts, and this drop continues after fertilization (Piko and Clegg, 1982), with different messages being degraded at widely diverse times. Some mRNA are rapidly degraded (ZP3), and degradation is delayed for others.

Activation of oocyte mRNA translation is regulated via the length of its poly (A) tail, involving cytoplasmic polyadenylation element binding protein (CPEB), cleavage and polyadenylation specific factor (CPSF) and poly(A) polymerase (PAP) (Dickson et al., 2001; Hodgman et al., 2001).

Specific kinetic changes in polyadenylation contribute to gene expression in embryos, and these changes influence further development (Brevini-Gandolfi et al., 1999; El Mouatassim et al., 1999; Brevini et al., 2002). Inadequate cytoplasmic maturation is linked to poor regulation of the mRNA polyadenylation process, and this leads to altered developmental competence (Brevini-Gandolfi et al., 1999; El Mouatassim et al., 1999; Brevini et al., 2002). The mRNA coding for Oct-4 (involved in early differentiation and totipotency), connexin-32 and -34 (important for blastocyst formation), for protection against free radicals and for polyadenylate polymerase are involved in this process of fine regulation. Injection of ‘good quality cytoplasm’ might possibly be beneficial by assisting the process of polyadenylation via injection of adenosine 3′-5′ biphosphate (PAP) and cytoplasmic...
polyadenylation element (CPE), and by elongating the polyA tail of some mRNA. On the other hand, some of the polyadenylated mRNA are submitted to a gradual reduction of their polyA tail (connexin-43 and Oct-4), and in this case, injection of exogenous factors could disturb the developmental process by introducing asynchrony into the embryonic cell cycle. Extra foreign cytoplasm might interfere more specifically during the maternal-to-zygotic transition cycle, with detrimental consequences. On the basis of mRNA status in recipient and donor cytoplasm, cytoplasmic transfer is of questionable value.

Conclusion

Based on the observations of Muggleton-Harris and Whittingham (1982) in the mouse, the concept of ooplasmic transplantation is currently very interesting. However, the indications for applying this technology in human clinical practice need to be clearly defined. Although there may be theoretical benefits of providing ‘appropriate’ but unidentified factors and/or presumably healthy mitochondria, the potential side-effects of these techniques must not be neglected. Relevant research concerning the role of apoptosis and the expression of apoptosis-related genes in oocyte and preimplantation embryo, with particular attention to mitochondria, could certainly be of benefit to infertile couples in the near future. At present, in the absence of validation by proper cell culture experiments or detailed animal research, the application of such therapies in humans is difficult to justify.

Furthermore, the paternal contribution to successful embryogenesis must not be ignored (Ménézo and Dale, 1995). The marked effect of sperm DNA fragmentation on embryo developmental potential and unexplained recurrent pregnancy loss is now well documented (Evenson et al., 2002; Carrell et al., 2003). Several studies have provided evidence to support the conclusion that sperm contain a complex repertoire of mRNA. Ostermeier et al. (2002) have recently demonstrated a biochemical contribution of the male gamete and its complementary role in fertilization and preimplantation embryogenesis. On this basis, it is difficult to assume that faulty early preimplantation embryo development (i.e. fragmentation) could be overcome by the addition of extra foreign ooplasm alone.

The treatment of infertile patients based upon their aetiology can be enhanced by an extensive understanding of the basic physiology–biochemistry of the human oocyte and early embryo. Before embarking upon ‘surgery and grafts’ in preimplantation embryos, it is now time to consider improving our knowledge concerning embryo metabolism and its relation to culture conditions based upon facts and evidence. This raises the question of how suitable oocytes for donating ooplasm should be identified or defined. It is obvious that criteria that will allow this evaluation are not yet available for clinical IVF, and will only be elucidated through fundamental basic research.

Aberrant methylation patterns at the 2-cell stage in mice have recently been identified under conditions of ‘ordinary’ in vitro culture (Shi and Haaf, 2002). In humans, major birth defects have been observed after assisted reproduction treatment by Hansen et al. (2002), but more importantly, an intriguing excess of imprinting anomalies in children conceived by IVF and/or ICSI has been reported (Cox et al., 2002; DeBaun et al., 2003; Gicquel et al., 2003; Maher et al., 2003; Orstavik et al., 2003; Powell, 2003). This reinforces the need for continuing evaluation of our ongoing technologies, and further questions the justification for more aggressive microsurgical techniques (Gosden et al., 2003).

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